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RESUMO

O herpesvírus murídeo do tipo 4, MuHV-4, é um gama-herpesvirus capaz de estabelecer infecções latentes em tecidos linfóides. Tal como outros herpesvírus, o MuHV-4 possui duas fases de expressão génica - a lítica e a latente – o que lhe permite estabelecer infecções crónicas durante toda a vida do hospedeiro. Durante a latência, o genoma de MuHV-4 persiste no núcleo de células latentemente infectadas sob a forma de episoma, não havendo produção de viriões. MuHV-4 estabelece latência maioritariamente em células B que se encontram em proliferação em centros germinais, que por sua vez dão acesso às células B de memória de longa duração.

Durante a infecção latente, o MuHV-4 expressa a proteína mLANA que se liga aos elementos repetitivos do genoma do vírus e aos cromossomas mitóticos do hospedeiro, permitindo a segregação do episoma em células em divisão. Além do seu papel fundamental na manutenção do episoma viral, mLANA é também uma proteína reguladora da transcrição, modulando substratos celulares, tais como NF-kB e MYC, sendo este último necessário para o desenvolvimento e manutenção de centros germinais. O mecanismo envolve a formação do complexo de ligase de ubiquitina EC_5S mediado por um motivo SOCS-box-like presente em mLANA. O complexo EC_5S^{mLANA} interage com RelA, um dos membros da família NF-kB, promovendo a sua poli-ubiquitinação e consequente degradação pelo proteossoma. O complexo EC_5S^{mLANA} também promove a poli-ubiquitinação de MYC mas contrariamente ao que acontece com NF-kB que é degradado, MYC é estabilizado durante o ciclo celular, levando a uma linfoproliferação. Deste modo, mLANA tem efeitos antagónicos sobre os dois factores de transcrição - inibe a actividade transcricional de NF-Kb mas activa a actividade transcricional de MYC. Portanto, ao sequestrar o controlo pós- transcricional de MYC, mLANA aumenta o número de células B infectadas nos centros germinais.

Numa tentativa, por parte de investigadores do nosso laboratório, para localizar a região de mLANA responsável pela interacção com os alvos, foi construída uma série de mutantes com delecções ao longo de *mLANA*. Embora o mapeamento tenha sido inconclusivo, o mutante D2 demonstrou aumentar a actividade E3 ligase de ubiquitina de mLANA resultando na estabilização de MYC e degradação proteossomal de NF-kB.

Em consequência do resultado supramencionado, o objectivo deste projecto foi definido: investigar o papel oncogénico da modulação de MYC durante a infecção por

gamma-herpesvirus. Para tal, dois vírus MuHV-4 recombinantes foram construídos, vD2 com uma delecção (Δ11-20) em mLANA que aumenta o efeito modulatório sobre MYC e vD2L92A que combina a mutação anterior com uma mutação em M2, uma proteína que se localiza predominantemente no citoplasma e membrana celular de células infectadas, e que possui um epítopo de células T restrito a H2-k^d que, sabe-se que induz uma linfoproliferação persistente em centros germinais. A construção dos vírus recombinantes foi feita através da mutagénese de BAC. Esta técnica permite a manutenção do genoma viral como BAC em *E. coli* e mutagénese do genoma através de recombinação homóloga. A confirmação da integridade das mutações em mLANA foi feita através de sequenciação ao longo da região mutada. A estabilidade do genoma viral clonado como plasmídeo BAC foi confirmada por digestão com as enzimas de restrição EcoRI e BamHI e os seus perfis de restrição foram comparados com os do MuHV-4 BAC. Após a identificação de BACs recombinantes, reconstruíram-se os vírus recombinantes em BHK-21 com posterior passagem por células NIH Cre 3T3 de forma a extrair as sequências BAC. Foi demonstrado que estas últimas sequências podem atenuar o crescimento dos vírus *in vivo*.

Para a produção de *stocks* virais, infectaram-se células BHK-21 com uma multiplicidade de infecção baixa de forma a não se produzirem vírus defectivos e os seus títulos foram determinados através de um ensaio de suspensão. Este ensaio consiste em diluições seriadas das suspensões virais com posterior adição de um número conhecido de células permissivas, células BHK-21. Após um período de incubação de 4 dias os títulos são calculados através da contagem de unidades formadoras de placas e tendo em conta o número de células permissivas adicionado.

Seguidamente foi avaliada a cinética da replicação viral dos vírus recombinantes através de curvas de crescimento *multistep*. Ambos os vírus demonstraram uma cinética semelhante à do vírus MuHV-4, demonstrando que as mutações introduzidas não afectaram a replicação lítica *in vitro*.

Os efeitos na fase latente de infecção das mutações introduzidas foram testados com um ensaio de reactivação *ex vivo*, em que ratinhos BALB/c foram infectados intranasalmente com MuHV-4 e as versões recombinantes. O ensaio consiste numa co-cultura de suspensões unicelulares provenientes de baços de animais infectados com fibroblastos permissíveis. A presença de vírus latentes na população inicial de esplenócitos resulta num efeito citopático nas camadas de fibroblastos e, a não ser que vírus infecciosos estejam presentes na altura da recolha dos baços, o efeito citopático é somente resultado da reactivação de vírus latentes. A capacidade de estabelecer e expandir uma infecção latente no baço foi avaliada aos

dias 14, 21 e 50 pós-infecção. Tanto vD2 como vD2L92A demonstraram um severo deficit no estabelecimento e manutenção de uma infecção latente. Enquanto animais infectados com o vírus D2 ainda exibiram uma dose latente baixa, com vírus latentes um pouco acima do limite de detecção, os vírus latentes D2L92A estiveram sempre abaixo do nível de detecção.

A proteína mLANA possui um sinal de localização nuclear putativo e, embora a mutação mLANA-D2 não tenho sido nesse local, pode ter modificado a estrutura da proteína e impedido a sua localização nuclear. Para tal, realizou-se uma imunofluorescência em células A20 transfectadas com plasmídeos mLANA-D2-GFP. Os resultados demonstraram que mLANA-D2 localiza-se no núcleo em células transfectadas.

A capacidade de expressão dos mutantes de mLANA também foi avaliada, através de experiências de *immunoblotting*. Para tal infectaram-se células BHK-21 com o vírus WT e as versões recombinantes e os resultados revelaram que ambos os vírus, vD2 e vD2L92A expressavam mLANA equivalentemente e que o fenótipo do ensaio de reactivação *ex vivo* não foi devido à falta da expressão da proteína viral.

O facto de os vírus recombinantes de MuHV-4 não conseguirem estabelecer e manter uma infecção latente, demonstra que as mutações introduzidas não são ideais para induzir linfoproliferação em centros germinais e desse modo obter um modelo de estudo para o desenvolvimento de alvos terapêuticos para doenças associadas a gamma-herpesvirus. Assim, é necessário desenvolver novas estratégias para a construção de vírus recombinantes capazes de manter uma infecção latente persistente.

Contudo, também seria interessante desenvolver estratégias de forma a descortinar a razão pela qual ambos os vírus recombinantes demonstraram um défice muito acentuado no estabelecimento e manutenção da latência. A razão pode ser devida à incapacidade da mLANA mutada em manter o seu papel putativo na manutenção do episssoma. A resolução deste problema pode contribuir grandemente para actuar sobre doenças crónicas causadas por gamma-herpesvirus.

Palavras-chave: mLANA, NF-Kb, MYC, ensaio de reactivação *ex vivo*, défice de latência.

ABSTRACT

Murid herpesvirus-4 (MuHV-4) is a gamma-2-herpesvirus capable of establishing latent infections in lymphoid tissues. During latency, MuHV-4 expresses mLANA, which mediates episome persistence, tethering viral genome to mitotic host chromosomes. Moreover, mLANA has been implicated in the modulation of the cellular transcription factors NF-kB and MYC by assembling and EC_5C^{mLANA} ubiquitin-ligase complex. EC_5C^{mLANA} targets NF-kB family member RelA for poly-ubiquitination and subsequent proteosomal degradation. EC_5C^{mLANA} also mediates MYC poly-ubiquitination but this leads to its turnover and stability. Thus, mLANA by increasing MYC stability through the cell cycle, subsequently potentiates lymphoproliferation and disease.

The aim of this project was to investigate the oncogenic role of MYC modulation during gammaherpesvirus infection. To this end, two recombinant MuHV-4 viruses were constructed: vD2 with mutations in mLANA that increase the modulation effect on MYC, and vD2L92A combining the previous mutation with a mutation in H2- k^d-restricted T cell epitope which induce persistent lymphoproliferation in germinal centres. Recombinant viruses were engineered by mutagenesis of the viral genome cloned as a BAC and tested on their capacity to establish and expand a latent load in the spleen and their ability to maintain a long-term persistence. Both recombinant viruses displayed a major deficit in splenic latency, incapable of establishing and maintaining a latent infection. The introduced delection in mLANA did not affected its nuclear localization as well its expression as it was assessed by immunofluoresce assay and immunoblotting, respectively. These results suggested that the mutations in mLANA somehow rendered recombinant viruses inaptitude to attach to host chromosomes and thus maintain a latent infection.

The overall results showed that vD2 and vD2L92A are not useful to study the molecular pathways critical for virus-induced lymphoproliferation. New strategies must be developed in order to potentiate tumorigenesis.

Keywords: mLANA, NF-Kb, MYC, infectious center assay, latency deficit

ABBREVIATIONS

BAC	Bacteria artificial chromosome
BHK	Baby hamster kidney
CTL	Cytotoxic $CD8^+$ T lymphocyte
DC	Dendritic cell
DBD	DNA-binding domain
DMEM	Dulbecco´s modified Eagle´s medium
EBV	Epstein-Barr virus
ECS	ElonginC-Cullin32/5-SOCS-box ubiquitin-ligase complex
ERK	Extracellular signal-regulated kinase
GC	Germinal centre
GFP	Green fluorescent protein
GMEM	Glasgow minimum essential medium
GSK	Glycogen synthase kinase
HCMV	Human cytomegalovirus
HEK	Human embryonic kidney
HHV-6	Human herpesvirus-6
HHV-7	Human herpesvirus-7
HHV-8	Human herpesvirus-8
HSV-1	Herpes simplex virus-1
HSV-2	Herpes simplex virus-2
kLANA	Kaposi latency-associated nuclear antigen
KS	Kaposi Sarcoma
KSHV	Kaposi´s sarcoma-associated herpesvirus
LANA	Latency-associated nuclear antigen
LB	Luria Bertani
mLANA	Murid latency-associated nuclear antigen
MOI	Multiplicity of infection
MuHV-4	Murid herpesvirus-4
NF-Kb	Nuclear factor-kappa B
NLS	Nuclear localization signal
ORF	Open reading frame
PBS	Phosphate buffered saline
PFU	Plaques forming unit
SCF	Skp1-Cullin1-F-box ubiquitin-ligase complex
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SOCS	Suppressors of cytokine signaling
TR	Terminal repeats
VZV	Varicella-zoster virus
WTmLANA	Wild-type mLANA

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1. INTRODUCTION

1.1 Herpesviruses

Herpesviridae family consists of enveloped viruses with large double-stranded DNA genomes that range from 120 to 250 kb. This family is ancient and widely disseminated in nature with members found in a large spectrum of vertebrate species (Barton *et al*., 2011). Herpesviruses are ubiquitous in the general population and remarkably well adapted to their hosts, a characteristic that is probably the result of a very long co-evolutionary history (Davison, 2002). Molecular phylogenetic studies suggest that herpesviruses speciation occurred at approximately the same time as did host speciation. As an outcome of this adaptation, herpesvirus infection of a natural immunocompetent host is rarely fatal (Davison, 2002). Herpesviruses have two alternative gene expression programs, a lytic and a latent phase, which allow herpesvirus to strategically establish life-long persistent infections in hosts. During the lytic replication, there is viral gene expression in a highly-ordered manner, being the viral genome replicated several times with the production of infectious virion progeny and the death of infected cells. Following lytic infection, the latent phase is established throughout the life of the host, with limited gene expression and without virion production enabling the virus to escape the host immune control (Wu *et al*., 2010)). This phase is characterized by sporadic reactivation episodes which ensure the transmission of infectious virions to new hosts as well as reinfection and establishment of a latent reservoir of infected cells for life (Cohen *et al*., 2003). In immunocompetent hosts, infections by several herpesvirus are usually asymptomatic or cause mild illness but in immunocompromised hosts can lead to fatal consequence (Wu *et al*., 2010).

The family *Herpesviridae* is divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Davison *et al*., 2009). Alpha-herpesvirus are neurotropic, establishing latency mainly in neurons of the sensory ganglia, beta-herpesvirus are the largest members of the herpesvirus family and are found latent preferently in hematopoietic stem cells and in cells of the myeloid lineage. Gammaherpesviruses are lymphotropic, establishing latency in B lymphocytes, being associated with lymphoid tissue (Davison *et al*., 2009). Until now, eight human herpesviruses have been identified: herpes simplex virus (HSV)-1, HSV-2 and

varicella-zoster virus (VZV) (*Alphaherpesvirinae*); human herpesvirus (HHV)-6 and HHV-7 and human cytomegalovirus (HCMV) (*Betaherpesvirinae*); Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), also known as HHV-8 (Wu *et al*., 2010).

1.1.1 Gammmaherpesviruses

Gammaherpesviruses are lymphotropic viruses widely spread in nature and although they are host-range specific, these viruses have been identified in a variety of mammalian species. Members of the gammaherpesvirus subfamily establish latent infections in lymphocytes, which can lead to the development of lymphoproliferative diseases and lymphomas as well as non-lymphoid cell tumors in immunocompromised hosts (Nash *et al*., 2001).

This subfamily is divided into two genera: gamma-1-herpesvirus, exemplified by Epstein-Barr virus (EBV) (Simas & Efstathiou, 1998) and gamma-2-herpesvirus, typified by the Kaposi's sarcoma-associated herpesvirus (KSHV) (Chang *et al*., 1994).

1.2 Kaposi's sarcoma-associated herpesvirus

Kaposi´s sarcoma-associated herpesvirus (or human herpesvirus 8) is the most recently discovered human herpesvirus (Chang *et al*., 1994) and has an etiologic role in Kaposi´s sarcoma as well in primary effusion lymphoma and multicentric Castleman's disease (Chang *et al*., 1994; Moore & Chang, 2003; Soulier *et al*., 1995). The presence of KSHV in the population is uncommon with approximately 1-5 % in North America and Northern Europe. In some Sub-Saharan Africa countries the seroprevalence rates exceed 60 % (Moore & Chang, 2003). Despite Kaposi´s sarcoma low malignant potential in immunocompetent individuals, because of the AIDS epidemic, KS became major contributor to morbidity and mortality in immunocompromised individuals (Verma & Robertson, 2003).

Similarly to other herpesvirus, KSHV employs a broad repertoire of immune evasion mechanisms in order to establish a persistent infection within its host. KSHV manage to do so by undergoing one of two alternative genetic life-cycle programmes after infection of host cells. The latent programme enables the virus to persist in a

relatively stable and immunological mode, whereas the lytic programme allows virions to be shed and transmitted to new hosts (Coscoy, 2007).

1.2.1 KSHV latency-associated protein (kLANA)

During the latent programme, KSHV genome persists as a multicopy, non-integrated, circular episome (Decker *et al*., 1996). This is accomplished by the expression of KSHV latency-associated nuclear antigen (kLANA or ORF73) which mediates the maintenance of the viral episome in the nucleus (Ballestas *et al*., 1999). kLANA tethers the viral genome to mitotic chromosome, which allows passive segregation of viral DNA to daughter nuclei and thus episome persistence in the progeny of dividing cells (Ballestas & Kaye, 2001; Garber *et al*., 2002). The C-terminus region of kLANA harbours a DNA-binding domain (DBD) that self-associates and binds to terminal repeats (TR) DNA sequences of the viral episome (Ballestas & Kaye, 2001; Schwan *et al*., 2000). The N-terminus region of kLANA is able to attach to chromosomes by binding nucleosomes through the folded region of histones H2A-H2B (Barbera *et al*., 2006). Another role for kLANA is in repressing the transcriptional activity of Rta, a KSHV immediate-early gene that activates lytic replication, maintaining latency (Lan *et al*., 2004). kLANA can also act like a nuclear regulator of gene transcription by interacting with several other cellular proteins thus regulating cell survival and growth (Verma *et al*., 2007). kLANA manage to do so by inactivating the tumor suppressor functions of p53 (Cai *et al*., 2006) and stabilizing and activating oncogene *c-Myc* (Liu *et al*., 2007).

The multiple functions of kLANA are critical for the maintenance and expansion of latency by playing several roles, namely viral genome maintenance, immune system evasion, controlling the switch between viral latency and lytic replication and promoting the survival and proliferation of latently infected cells.

1.3 Murid herpesvirus-4

Murid herpesvirus-4 (MuHV-4, also referred to as MHV-68) was first isolated from bank voles (*Clethrionomys glariolus*) captured in Slovakia and is a natural pathogen of wild rodents (Blaskovic *et al*., 1980). It readily infects laboratory mice (*Mus*

musculus), representing a tractable model system for studying gammaherpesvirus pathogenesis and enabling the assessment of molecular mechanisms involved in virus-host interaction (Simas & Efstathiou, 1998). MuHV-4 genome consists of 118 kb of unique sequence DNA with a G+C content of 46%, flanked by multiple copies of 1, 2 kb terminal repeat regions (Efstathiou *et al*., 1990). It has approximately 80 open reading frames (ORFs) which are collinear and homologous of other gammaherpesviruses, namely KSHV, and has several genes encoding functionally homologous to cellular proteins (Nash *et al*., 2001; Virgin *et al*., 1997). These characteristics closely relate MuHV-4 to gamma-2-herpesvirus genus, like KSHV (Virgin *et al*., 1997).

Similarly to other gamma-2-herpesvirus, MuHV-4 establishes a lifelong chronic infection in lymphoid tissues (Sunil-Chandra *et al*., 1992b), in which its genome persists as an episome in latently infected cells. Mice inoculated via the olfactory neuroepithelium (Milho *et al*., 2012) with MuHV-4 establish an acute phase of virus replication in the lungs involving infection of alveolar epithelial cells (Stewart *et al*., 1998; Sunil-Chandra *et al*., 1992a) which is resolved within 9 to 12 days post-infection (dpi) (Sunil-Chandra *et al*., 1992b). After the lytic infection, the virus is spread to lymph nodes via dentritic cells (DCs) (Gaspar *et al*., 2011) and reaches the spleen by infecting marginal zone (MZ) macrophages which then pass it to MZ B cells (Frederico *et al*., 2014).

The peak of latency is accompanied by a transient splenomegaly (Sunil-Chandra *et al*., 1992a) with the levels of latently infected cells decreasing thereafter but remaining steady (Cardin *et al*., 1996; Simas & Efstathiou, 1998). Although other cell types have been implicated in latency – alveolar epithelial cells, dendritic cells and macrophages – MZ B cells appear to be the main route in which MuHV-4 establishes latency in B cells that are proliferating in a germinal centre (GC), which in turn give access to the virus to the long-lived memory B cell pool (Frederico *et al*., 2014; Marques *et al*., 2003). With an equal contribution, these populations of cells have key roles in both in the establishment and maintenance of viral latency (Flaño *et al*., 2002).

Human gammaherpesviruses, such as EBV and KSHV, do not infect a suitable laboratory animal, since they are species-specific viruses. The result is that several aspects of their *in vivo* pathogenesis have remained unknown. With the discovery of MuHV-4 infecting mice, a tractable model system to investigate gammaherpesvirus has arisen, offering a unique opportunity to investigate immunological and virological aspects of gammaherpesvirus pathogenesis (Simas & Efstathiou, 1998).

1.3.1 MuHV-4 latency-associated protein (mLANA)

Similarly to KSHV, MuHV-4 encodes a latency-associated protein, a product of ORF73 gene. ORF73 protein (or mLANA) has 314 amino acid residues and has substantial homology in sequence and function to ORF73 protein of KSHV (Grundhoff & Ganem, 2003). Previous studies have shown that mLANA is required both *in vitro* (Habison *et al*., 2012) and *in vivo* (Fowler *et al*., 2003) for the establishment and maintenance of latency.

The crucial role of this protein was evidenced when mice infected with recombinant virus with mLANA disrupted could not establish splenic latency (Fowler *et al*., 2003). In a mouse model of infection, mLANA is selectively expressed in infected GC B cells (Marques *et al*., 2003). Similarly to KSHV LANA, mLANA was recently shown to act on terminal repeat (TR) elements of the MuHV-4 genome to mediate episome persistence, tethering viral episome to mitotic host chromossomes allowing episome segregation to daughter nuclei during cellular division (Habison *et al*., 2012). The C-terminal region of mLANA, comprising amino acid residues 140 to 263, has amino acid similarity to the kLANA DNA-binding domain (Correia *et al*., 2013).

Like KSHV LANA, mLANA is also a regulator of transcription. Rodrigues *et al.* have demonstrated that MuHV-4 LANA promotes poly-ubiquitination of cellular substrates, NF-kB and MYC, by interfering with their turnover, thus modulating their cellular transcription (Rodrigues *et al*., 2009; Rodrigues *et al*., 2013).

All this similar characteristics with KSHV LANA, make MuHV-4 LANA an excellent model for investigating episomal maintenance and establishment of viable latency *in vivo*.

1.3.2 Modulation of cellular gene expression by mLANA

Being obligatory intracellular parasite, MuHV-4 has evolved a repertoire of mechanisms to modulate specific host cellular genes to favor their own replication. MuHV-4 manage to do so through the activity of mLANA, which has been implicated to function as nuclear regulator of transcription and to interact with several cellular proteins to modulate host functions (Verma *et al*., 2007). One of such mechanisms

targets nuclear factor-kappa B (NF-kB), a family of ubiquitously expressed transcription factors. NF-kB family members can form homo- or heterodimers and bind to specific DNA sequences, kB sites, in the promoter region of a variety of genes to modulate their transcription/expression (Saccani *et al*., 2003). Genes regulated through NF-kB are involved in critical biological functions, including inflammation and apoptosis as well as cell proliferation and differentiation (Baldwin, 2001). Recently it has been shown that mLANA is capable of modulating the NF-kB signaling pathway. This mechanism involves the assembly of an EC_5S complex mediated by an unconventional SOCS-box-like motif (amino acid residues 199-215) present in mLANA. EC_5S^{mLANA} mimics the host ECS^{SOCS1} ubiquitin-ligase, targeting nuclear-activated RelA for poly-ubiquitination and subsequent proteasomal degradation, resulting in termination of NF-kB response (Rodrigues *et al*., 2009).

Another mechanism employed by MuHV-4 to promote its own life cycle is the stabilization of Myc through heterotypic poly-ubiquitination (Rodrigues *et al*., 2013).
MYC proto-oncogene family, which includes c-MYC, N-MYC and L-MYC, is essential for the control of normal cell proliferation, growth, survival and differentiation (Adhikary & Eilers, 2005). However, its deregulation results in an enlarge pool of proliferating, self-renewing cells which accelerate tumorigenesis events contributing to a wide variety of human cancers (Larsson & Henriksson, 2010). On the other hand, deregulation of MYC also triggers anti-tumorigenic responses including apoptosis, cellular senescence and DNA damage responses (Larsson & Henriksson, 2010).

Being MYC a global transcriptional regular it would be expected to be expressed at relatively high levels which it is not the case. MYC is tightly regulated both at the transcriptional and post-transcriptional levels, namely through poly-ubiquitination. In non-transformed cells, MYC is continuously subjected to ubiquitination and proteosomal-degradation, resulting in a highly unstable protein with a half-life of about 15-20 minutes (Gregory & Hann, 2000). The mechanism involves the interplay between phosphorylation at two specific residues and ubiquitination. Phosphorylation at serine (S) 62 by extracellular signal-regulated kinase (ERK) stabilizes MYC resulting in enhancement of its transcription activity. In contrast, phosphorylation of MYC at threonine (T) 58 by glycogen synthase kinase 3 (Gsk-3), which is dependent on previous phosphorylation of MYC at S62, leads to proteasomal degradation of MYC (Sears *et al*., 2000). This happens because of the assembly of homotypic poly-ubiquitination chains on MYC dependent in lysine (K) 48 linkage by SCF

(Skp1/Cul/Fbox)^{Fbw7}. MYC turnover by SCF^{Fbw7} is antagonized by polimeration of mixed heterotypic poly-ubiquitination chains via SCF$^{\beta\text{-}TrCP}$ on the N-terminus of MYC (Popov *et al*., 2010).

Thus SCF$^{\beta\text{-}TrCP}$ (Skp1-Cullin1-F-box) E3 ubiquitin-ligase complex mediates MYC poly-ubiquitination leading to its turnover and stability, instead of targeting MYC to proteasomal degradation (Popov *et al*., 2010). The physiological relevance of regulating MYC activity is underscored by observations that in many B-cell lymphomas, point mutations at or near T58 occur that disrupt phosphorylation and subsequently proteasomal degradation, rendering MYC resistant to degradation by SCF^{Fbw7} (Bahram *et al*., 2000).

It is known that MYC is essential for the initiation and maintenance of GC (Calado *et al*., 2012; Dominguez-Sola *et al*., 2012) and MuHV-4 depends critically on the expansion of latently infected B-cell in GC reactions for host colonization. GC reactions exhibit two distinct morphological areas which include the dark zone (DZ) adjacent to the T cell zone and the light zone (LZ) contiguous to the splenic marginal zone. It has been shown that MYC expression is restricted to clusters of B-cells that initiate GCs and a small fraction of LZ B-cells. Furthermore ablation of MYC expression or activity blocks GC formation (Calado *et al*., 2012; Dominguez-Sola *et al*., 2012). So by hijacking the post-translational control of MYC, mLANA increases the number of MuHV-4 infected B-cells in the GC. mLANA manages to do so by acting like β-TrCP adaptor protein, binding to MYC and promoting its poly-ubiquitination and subsequent stabilization. Thus, mLANA increase MYC stability through the cell cycle and subsequently lymphoproliferation and disease (Rodrigues *et al*., 2013).

In an attempt to map the region of mLANA responsible for interaction with targets, a collection of mutants was generated with deletions across mLANA (S. Cerqueira unpublished data). Mapping was inconclusive but D2 (Δ11-20) was shown to increase E3 ubiquitin-ligase activity of mLANA resulting in stabilization of MYC and proteosomal degradation of NF-kB.

1.4 MuHV-4 M2 Protein

M2 gene is located at the left end of MuHV-4 genome, a region containing four MuHV-4 unique ORFs (M1-4) as well as eight viral tRNAs and thus, M2 was initially

classified as a latency-candidate gene on the basis of its genomic position (Virgin *et al*., 1997). The M2 transcript produces a 192 amino acid-long protein (Husain *et al*., 1999) and does not have homology to any cellular or viral proteins. It has been shown to act as an adaptor protein that binds SH2 domains through two phosphor-tyrosine motifs and to SH3 domain through PxxP motifs (Pires de Miranda *et al*., 2008; Rodrigues *et al*., 2006). Later it was confirmed that M2 is a latency-associated gene by demonstrating its expression in latently infected murine B cell lymphoma line S11 and in splenocytes of intranasally infected wild type mice during the establishment of latency (Husain *et al*, 1999).

M2 gene is expressed while establishment of latency in the spleen in several B cell subsets, namely germinal center and during infection in GC B cells (Flaño *et al*., 2002; Marques *et al*., 2003). To further characterize the role of M2 expression in the overall establishment of a normal load, M2 recombinant viruses were used. Jacobi et al demonstrated that following intranasal infection of a recombinant virus without a functional expressing M2, there was no change in the acute phase of virus replication compared to that WT virus, but caused a severe decrease in the establishment of latency in the spleen and a defect in reactivation from latency (Jacoby *et al*., 2002). In 2003, Macrae et al. besides corroborating the above results, showed that disruption of M2 did not affected the ability of the virus to reactivate from latency, that the mutation of M2 did not negatively affect the establishment of long-term latency and that in B cells, M2 protein is localized predominantly in the cytoplasm and plasma membrane (Macrae *et al*., 2003). The studies demonstrated that M2-deficient mutants replicate normally in tissue culture and display normal acute phase replication kinetics *in vivo*.

Studies performed by our group showed that the M2 gene product is required for efficient colonization of splenic follicles but is dispensable for the expansion of latency infected GC B cells at early times post-infection (Simas *et al*., 2004). An epitope was identified in the M2 protein ($M2_{84-92}/K^d$, residues GFNKLRSTL) that is actively recognized by $CD8^+$ T cells from H2-K^d infected BALB/c mice and a CTL line capable of recognizing this epitope was able to kill S11 cells, meaning that M2 expressing cells constitute a target for immune response during latency (Husain *et al*., 1999). Usherwood et al (2000), demonstrated that $M2_{91-99}/K^d$ T cell response is B cell dependent, transient and apparently prompted by the rapid increase in latently infected cells around day 14 after intranasal infection. Adoptive transfer of M2-specific $CD8^+$ T cells impaired the expansion of latency normally occurring in the spleen 14 days post-

infection, supporting the idea that this latent antigen-specific immune response plays a role in controlling the initial “burst” of latently infected cells (Usherwood *et al*., 2000). Investigations from our group revealed that a single $CD8^+$ T cell epitope sets the long-term latent load (Marques *et al*., 2008) By mutating M2 anchor residues to alanines, either the phenylalanine at position 85 ($vM2_{F85A}$) or the leucine at position 92 ($vM2_{L92A}$), the generation of the H2-K^d-GFNKLRSTL-specific $CD8^+$ T cells was prevented. The acute latency phenotype was reproduced but not the long-term sustained proliferation in GC B cells, indicating that this phenotype was due to the disruption of an epitope that is critical for CD8+ T cell-mediated control of latency in infected cells. Marques *et al* (2008), showed that disrupting $CD8^+$ T cell recognition of M2 allowed more extensive proliferation of latently infected B cells. When M2 is not expressed, $CD8^+$ T cells are not primed efficiently and cannot control the amplification of latency in GC B cells (Marques *et al*., 2008).

2. AIMS OF THE PROJECT

The aim of this project was to investigate the oncogenic role of MYC modulation during gammaherpesvirus infection. To achieve this, recombinant MuHV-4 bearing mutations in mLANA that increase the modulation effect on MYC by over 10-fold were produced and phenotyped during infection of laboratory mice. To potentiate the genesis of tumor development, recombinant viruses combining the previous mutations with mutations in as H2-K^d-restricted T cell epitope, which was shown to induce persistent lymphoproliferation in germinal centres were also generated.

The overall goal is that such study will reveal molecular pathways critical for virus-induced lymphoproliferation that can constitute future basis for the development of pharmacological targets to resolve virus-associated disease.

3. MATERIALS AND METHODS

3.1 Plasmids

pCMV-Myc enconding mLANA-D2 was generated by site-directed mutagenesis using QuickChange II XL kit (Agilent Technologies) according to the manufacturer's instructions (S. Cerqueira).

To generate pCMV-Myc-mLANA-D2-maxGFP, pCMV-Myc-maxGFP and pCMV-Myc-mLANA-D2 were digested with EcoRI to excise maxGFP (0.7 kb) and to linearize the plasmid encoding mLANA-D2 (4.8 kb). The digested fragments were gel purified using Agarose Gel DNA Extraction Kit (Roche) according to the manufacturer's instructions, and ligated. Competent *E. coli* DH5α were transformed by the heat shock method and colony PCR was performed using primers CMV F (5'-GATCCGGTACTAGAGGAACTGAAAAAC-3') and CMV R (5'-AATAGCATCACAAATTTC-3') to confirm ligation of the maxGFP. PCR products with the insert were purified using High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions. pCMV-Myc-mLANA-D2 fused with maxGFP was then digested with PstI to confirm the direction of maxGFP insert in the pCMV-Myc-mLANA-D2 (1980 bp).

3.2 Bacteria

Competent *E. coli* harboring BAC WT or BACL92A (Marques *et al*., 2003) were used during the generation of recombinant viruses.

3.3 Mice

BALB/c mice used in this study were obtained from Charles River. Animals were housed and subjected to experimental procedures in specific pathogen-free conditions, at the Instituto de Medicina Molecular animal facility, Lisbon, Portugal.

3.4 Cell culture and DNA Transfections

NIH 3T3 – Cre (mouse embryonic fibroblasts) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal calf serum, 2 mM *L*-glutamine and 100 U/mL penicillin-streptomycin. Cre 3T3 cells were used during the construction of recombinant MuHV-4 viruses to remove the *lox*P-flanked BAC cassette. Baby hamster kidney (BHK-21) fibroblasts cells were cultured in Glasgow minimum essential medium (GMEM) supplemented as above plus 10 % tryptose phosphate broth. These cells were used for growing and titrating viral stocks, *ex vivo* reactivation and plaque assays, *in vitro* multi-step growth curves and analysis of mLANA expression.

Transiently transfection of BHK 21 cells with plasmid DNA was performed with X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science) according to manufacturer's instructions. A20 murine B lymphoma cells were cultured in RPMI medium supplemented with 10 % foetal calf serum, 2 mM *L*-glutamine and 100 U/mL penicillin-streptomycin. A20 cells were used for immunofluorescence. Transfection of A20 cells was accomplished by electroporation (270 V, 500 F), using a Bio-Rad gene pulser. In all transfections, an empty vector was used to normalize the total amount of plasmid DNA. All cell cultures were grown in a tissue culture incubator at 37 ºC under 5 % CO2.

3.5 Transformation of competent cells

Competent *E .coli* DH10B harboring BAC WT or BACL92A (Marques *et al*., 2003) were transformed by the heat shock method. Each aliquot was incubated on ice for 30 min with 1 µg of plasmid DNA (shuttle plasmid), followed by heat shock for 45 sec at 42 ºC and subsequently chilled on ice for 2 min. 500 µL of SOC medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) were added to each vial and cells were incubated for 1 h at 30 ºC. Cells were then spread onto LB agar plates containing chloramphenicol (17 µg/mL) and kanamycin (30 µg/mL).

Competent *E. coli* DH5α were transformed as above and spreaded on LB agar plates containing ampicillin (100 µg/mL).

3.6 Generation of recombinant virus

MuHV-4 recombinant viruses were generated by mutagenesis of the viral genome cloned as a bacterial artificial chromosome (BAC) (Adler *et al.*, 2000). D2 deletion (mLANA nucleotides 11-20) was introduced by PCR on BamHI-G genomic clone using QuickChange II XL (Agilent Techonologies). Recombinant BamHI-G fragment was subcloned into the BamHI site of pST76K-SR shuttle plasmid and transformed into an *Echerichia coli* strain (DH10B) containing the wild-type MuHV-4 BAC or MuHV-4 BACL92A (S. Cerqueira). Bacteria were plated on LB plates containing kanamycin (shuttle plasmid resistance marker) and chloramphenicol (BAC plasmid resistance marker) and incubated overnight at 30 ºC, since the shuttle plasmid has a temperature-sensitive origin. The shuttle plasmid encodes the recA protein that promotes the complete integration of the shuttle plasmid into the viral BAC genome by homologous recombination (Adler *et al.*, 2003). Bacteria containing the cointegrate were selected by incubation overnight at 42 ºC on LB plates containing both antibiotics. Clones were replated on chloramphenicol-containing LB plates and grown for one day at 30 ºC. Under these conditions, cointegrates can spontaneously resolve by homologous recombination to either wild-type or mutant BAC plasmid. Bacteria harboring a resolved cointegrate were selected by replating on LB plates containing chloramphenicol and 5 % sucrose (counterselection against SacB gene encoded by the shuttle plasmid) and grown at 30 ºC. Colonies were plated in parallel on kanamycin- and chloramphenicol-containing plates and incubated overnight at 37 ºC.

3.7 Small scale BAC plasmid preparations

For small BAC plasmid preparations chloramphenicol-resistant kanamycin-sensitive colonies were inoculated in 10 mL of LB broth containing chloramphenicol (17 µg/mL) with shaking for 12-18h at 37 ºC. Bacteria were pelleted by centrifugation at 1575 xg for 10 min. at 4 ºC. Pellet was resuspended in 200 µl of buffer S1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA; from Qiagen) by pipetting. 300 µl of buffer S2 (200 mM NaOH, 1 % SDS; from Qiagen) were added, suspension was gently mixed by inversion and left at RT for 5 min. 300 µl of chilled buffer S3 (3.0 M KOAc pH 5.5; from Qiagen) were added, tubes were inverted to mix the solution, incubated on ice for 15 min. and

centrifuged for 10 min at 16200 xg at 4 ºC. Supernatant was then transferred to a new tube, 1 ml of phenol/chloroform (1:1) was added and mixed by inversion. Mixture was left at RT for 5 min. BAC plasmid DNA was pellet by centrifugation for 30 min. at 16200 xg at 4 ºC. Supernatant was discarded and the pellet was washed with 70 % ethanol, centrifuged another 10 min., drained and air dried. DNA was resuspended in 50 µl of MiliQ water and stored at 4 ºC.

3.8 Identification of recombinant BAC clones

To identify mutant BAC clones, the region containing the mutation was amplified by PCR with primers ORF73Rb (5'-AAAGCGGCCGCTGTCTGAGACCCTTGTCC-3') and CS7 (5´-AAAGAATTCTAATGCCCACATCC-3') and identified by DNA sequencing.

Recombinant BAC clones were then isolated by large-scale BAC preparations using Nucleic Acid and Protein Purification kit (Macherey-Nagel), according to manufacturer's instructions. Briefly, single colony bacteria were inoculated in 250 mL of LB broth supplemented with chloramphenicol and incubated with shaking for 12-18h at 37 ºC. Bacteria were then pellet by centrifugation at 3214 xg for 15 min. at 4 ºC and BAC plasmid DNA was isolated using the above mentioned kit. DNA was then resuspended in 100 µl of MiliQ water and stored at 4 ºC. The integrity of recombinant BAC clones was confirmed by restriction digestion with BamHI and EcoRI and restriction profiles were assessed by agarose gel electrophoresis with 0.8 % agarose in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

3.9 Reconstitution of MuHV-4 virus from BAC DNA

For virus reconstitution, recombinant BAC DNA (1 µg) was transfected into BHK-21 cells ($5x10^5$) using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science), according to manufacturer's instructions. When approximately 50 % cpe was visible, cells and media were harvested and subjected to a freeze-thawing cycle to disrupt the cells and stored at -80 ºC. The obtained viral aliquots constituted the BAC^+ master stock. The loxP-flanked BAC cassette was removed by viral passage through NIH Cre 3T3 cells and limiting dilution to obtain GFP-negative viruses.

3.10 Virus working stocks

For virus working stocks, BHK-21 cells were infected at a low MOI (0.001 pfu/cell) in 175 cm^2 culture flasks. After an incubation period of 4 days, cells and supernatant were transferred to 50 mL falcon and centrifuged at 290 xg, 4 ºC for 5 min. Cell-associated viruses were resuspended in 2 mL of complete GMEM, subjected to freeze-thawing and kept at -80 ºC in 200 µl aliquots. Supernatant-associated viruses were centrifuged at 15000 xg for 2h at 4 ºC and the pellet was resuspended in 2 mL of complete GMEM. 100 µl aliquots were made and store at -80 ºC. Virus titers were determined by plaque assay.

3.11 Plaque Assay (Suspension Assay)

Virus titers were determined by plaque assay (or suspension assay). $5x10^5$ BHK-21 cells were added to a serial 10-fold dilution of virus suspensions in 2 mL of complete GMEM and incubated for 1h. 3 mL of complete GMEM were then added and the suspension was plated out into 6 well-plates. After 4 days incubation at 37ºC and 5 % CO_2, cell monolayers were fixed with 10 % formaldehyde in PBS and stained with 0.1 % toluidine blue. Viral plaques were counted using a magnifier lenses and virus titers were calculated from the number of viral plaques on duplicate dishes.

3.12 *In vitro* Multi-Step Growth Curves

Low multiplicity growth curves were performed on confluent BHK-21 cell monolayers in 24-well plates. BHK-21 cells ($5x10^4$ cells) were infected with the WT virus and mutant versions at a multiplicity of infection (MOI) of 0.01 pfu/cell, in a total volume of 200 µL, in each well. Virus was then allowed to adsorb for 1h at 37 ºC. Cells were washed with 500 µL of PBS and resuspended in 1 mL of fresh GMEM. At each different time point after infection, cells and supernatant were harvested, subjected to freeze- thawing and kept at -80 ºC. Virus titers were determined by suspension assay in duplicate.

3.14 *Infectious Centre Assay*

Infectious center assay is a well-established assay in which single cell suspensions are prepared from the harvested spleens of infected mice and co-cultured with permissive fibroblasts cells. The presence of latent virus in the initial splenocyte population is revealed by the observation of cytopathic effects (cpe) on the above cells.

Female BALB/c mice with 6 to 8 weeks old were inoculated intranasally with $1x10^4$ pfu in 20 µL of PBS with WT virus and mutant versions, under isofluorane anaesthesia. At days 14, 21 and 50 after infection, mice were sacrificed by inhalation of CO_2 and spleens were dissected into 5 mL of complete GMEM and kept on ice for the majority of the assay. Single splenocyte suspensions were obtain by mechanical disruption and filtered through a 100 µm cell strainer in order to remove stromal debris. Cells were then pellet by centrifugation at 217 xg for 5 min. at 4 ^oC. Supernatant was discarded and cells were resuspended in 1 mL of Red Blood Cells Lysis Buffer (154 mM ammonium chloride, 14 mM sodium hydrogen carbonate, 1 mM EDTA pH 7.3) and incubated on ice for 5 min. 10 mL of complete GMEM was added, cells were centrifuged and resuspended in 5mL of fresh medium. 2-fold and 10-fold serial dilutions of splenocyte suspensions were prepared, in duplicate, to a final volume of 2 mL in 12-well plates. $5x10^5$ BHK-21 cells in 4 mL of complete GMEM were plated in 6 cm^2 dishes and 1 mL of each splenocyte dilution was added. Plates were incubated for 5 days at 37 ^oC, 5 % CO_2, and then were fixed with 1 % formaldehyde in PBS and stained with 0.1% toluidine blue. Viral plaques were counted with the help of a magnifier lenses and infectious centers (pfu/spleen) were determinate in duplicate.

In order to confirm that the results obtained for each virus was a reflection of a truly latent infection; spleens were also analyzed for the presence of pre-formed infectious centers viruses in freeze-thawed splenocyte suspensions by plaque assay.

3.15 *Immunofluorescence analysis*

A20 cells grown on poly-L-lysine-coated coverslips were transiently transfected with 15 µg of pCMV-Myc-mLANAD2-max-GFP or pCMV-Myc-mLANA-GFP. After 24 h, cells were washed in PBS and incubated in fixative solution (2 % sucrose and 5 % formaldehyde in PBS) for 20 min. After three washes in PBS, cells were permeabilised

(0.1 % Triton X-100 in PBS) for 15 min, and then washed once in PBS. DNA was stained with DAPI solution (5 µg/mL) in order to the nucleus be visualized. Following staining, cells were once again fixed in fixative solution for 5 min. After three washes in PBS, coverslips were rinsed in distilled water and mounted onto microscope slides with Mowiol.

3.16 Immunoblotting

For analysis of mLANA expression, BHK-21 cells ($5x10^5$) were infected with MuHV-4 WT virus and recombinant virus at a MOI of 2.5 pfu/cell in a total volume of 750 µL. Viruses were allowed to adsorb for 1h at 37 ºC and then 2 mL of complete GMEM was added. Cells were incubated for 24 h at 37 ºC. After 24 h, cells were washed in PBS, harvested and transferred to a total volume of 1 mL. Cell suspension was centrifuged at 16200 xg, 10 min, 4 ºC. Cell pellets were lysed with 25 µL of Passive Lysis Buffer (Promega). Samples were cleared by centrifugation at 3700 xg, 2 min. at 4 ºC. 25 µL of supernatant were recovered and added to 25 µL of reducing Laemmli's buffer (2x) and heated at 100 ºC for 10min. Proteins were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1.5 mm mini-slab gels of Bio-Rad mini-protean II electrophoresis system. Resolving gel was prepared with 10 % polyacrylamide (Acrylamide:bis-acrylamide 37.5:1, Bio-Rad), 375 mM Tris-HCl (pH 8.8), 0.1 % SDS, 0.1 % APS and 0.04 % TEMED. Stacking gel was prepared with 4 % polyacrylamide, 125 mM Tris-HCl (pH 6.8), 0.1 % SDS, 0.1 % APS and 0.1 % TEMED. Electrophoresis was performed in running buffer (25 mM Tris-base, 192 mM glycine and 0.1 % SDS), at 120 V.

After resolved by SDS-PAGE, proteins were transferred to a nitrocellulose membrane using a Trans-Blot Cell (Bio-Rad), assembled according to manufacturer's instructions, in a standard transfer buffer (25 mM Tris-Base, 200 mM glycine and 10 % methanol) for 1h 30 min at 250 mA. Following electroblotting, any unoccupied protein binding sites were blocked by incubation of the membrane in a 5 % (w/v) skimmed milk powder in PBS-T (PBS with 0.05 % Tween 20) for 1h at RT on a shaking platform. mLANA protein was detected with anti-mLANA monoclonal antibody (1:1000 in blocking buffer) (Pires de Miranda *et al*, 2013) with incubation for 2 h, at RT on a shaking platform. The membrane was washed in PBS-T three times, 5 min. each with

incubation in a shaking platform to remove the unbound primary antibody. The primary antibody was detected by incubation of the membrane with horseradish peroxidase-conjugated anti-mouse IgG (1:5000 in blocking buffer) for 50 min. at RT, again in a shaking platform. The membrane was washed as described above and the proteins bands were visualized by chemiluminescence using ECL according to manufacturer's instructions.

Actin was detected with a rabbit anti-actin polyclonal antibody (Sigma).

4. RESULTS

4.1 Generation and characterization of recombinant MuHV-4 viruses

In order to investigate the oncogenic role of MYC, two mLANA mutants were generated. mLANA-D2 bearing a deletion in nucleotides 11-20, which increase E3 ubiquitin-ligase activity of mLANA, resulting in stabilization of MYC and proteosomal degradation of NF-kB and mLANA-D2L92A which combine the previous mutation with substitution of alanine by leucine at position 92 in the M2 anchor residues, using MuHV-4 BAC pHA3 (Adler *et al*., 2000). This technique allows the maintenance of the viral genome as a BAC in *E.* coli and the site-directed mutagenesis of the genome by homologous recombination (Adler *et al*., 2003). The generation and initial characterization of the recombinant viruses is described in detail in Materials and Methods. The confirmation of the integrity of the mLANA mutations was made through sequencing across the mutated region in the BAC plasmid. The MuHV-4 genome contains a number of repeats; the terminal repeats, an internal 100 bp repeat and an internal 40 bp repeat (Virgin *et al*., 1997) which may be prone to recombination events and although the viral BAC plasmids were maintain in recA-negative bacterial strain (DH10B), recombination between direct repeats may cause deletion in the copy number of the repeated sequences. Therefore, the stability of the viral genomes cloned as BAC plasmids were verified by digestion with EcoRI and BamHI and compared with MuHV-4 BAC (Figure 1). Identical restriction profiles indicate that no gross changes in genome occurred. Infectious viruses were reconstituted by transfecting the BAC-DNA into BHK-21 fibroblasts. The viruses were then sub-cultivated through Cre recombinase-expressing fibroblasts to remove the BAC vector sequences.

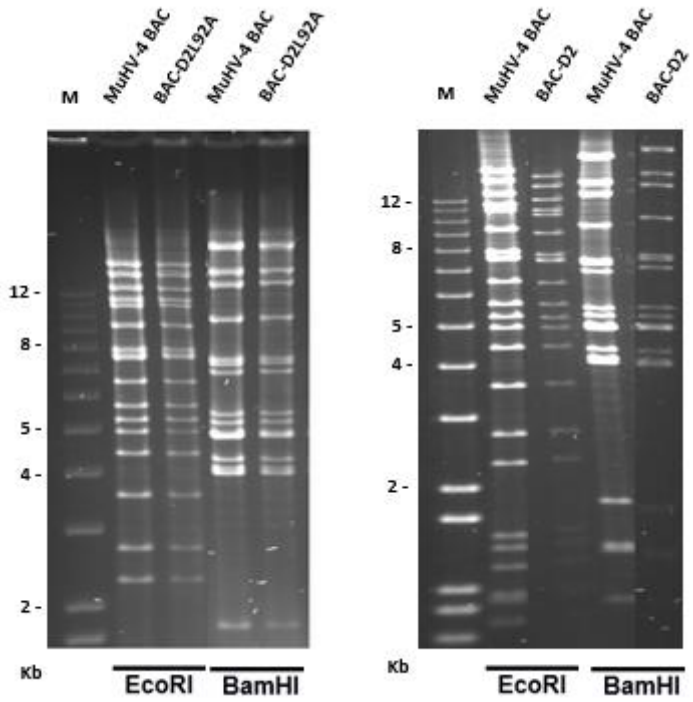

Figure 1. Stability of viral genomes cloned as BAC plasmids in *E. coli*. BAC plasmids were digested with EcoRI and BamHI restriction enzymes and restriction profiles were compared with ones of MuHV-4 BAC. Marker sizes (in kilobase pairs) are indicated on the left. The analysis of the clones is shown on a Gel Red-stained agarose gel.

4.2 Recombinant MuHV-4 viruses display normal growth in vitro

The kinetics of viral replication of the recombinant viruses was assessed by a multisptep growth curves in permissive BHK-21 fibroblasts, infected at a MOI of 0.01 pfu/cell. It was demonstrated that mLANA is not essential for *in vitro* replication of MuHV-4, since mLANA-negative viruses displayed normal growth (Fowler *et al*., 2003; Moorman *et al*., 2003). On the other hand, M2 was also demonstrated to be dispensable for lytic replication *in vitro*, studies have showed that M2-deficient mutants display normal acute phase replication kinetics (Jacoby *et al*., 2002; Macrae *et al*., 2003; Simas *et al*., 2004). All viruses replicated equivalently to the WT at all-time points measured.

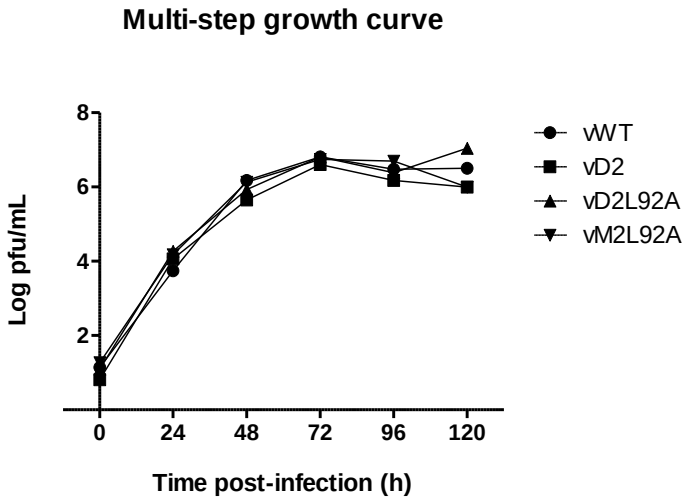

Figure 2. Recombinant MuHV-4 viruses show normal *in vitro* replication. Multistep growth curves were constructed by infection of BHK-21 cells with the indicated virus at a multiplicity of infection of 0.01 pfu/cell and washed in PBS. At the indicated times post-infection, samples were harvested, freeze-thawed and titers were determined by plaque assay on monolayers of BHK-21 cells.

4.3 MuHV-4 LANA-D2 and LANA-D2L92A have a deficit in splenic latency

In order to phenotype and analyze the effect of the introduced mutations in MuHV-4 latent phase of infection, BALB/c mice were infected intranasally with vWT, mutante versions and vM2L92A, which served as control for the M2 mutation, and the latent load in the spleen was examined by quantification of *ex vivo* reactivation-competent viruses by infectious center assay. This assay consists of co-cultivation of single cells suspensions prepared from the harvested spleens with permissive fibroblasts cells. The presence of latent viruses in the initial splenocyte population results in cytopathic effect (cells lysis plaques) on fibrobaslts monolayers. Unless formerly infectious viruses are present at the time of harvest, the cytopathic effect (cpe) can only result from viral reactivation latency. The four viruses were evaluated at days 14, 21 post-infection (pi) for their capacity of establish and expand a latent load in the spleen and day 50 pi for their ability to maintain a long-term persistence. The results are shown in Figure 3. The WT virus presented the expected peak of infection at day 14 pi, with latent infection persisting through day 21 to become undetectable by day 50 pi. The vM2L92A also displayed an expected pattern of infection, with the presentable epitope having a major impact on the long-term viral load as can be seen at day 50 pi (Marques *et al*., 2008). In contrast, both D2 and D2L92A viruses showed a major deficit in establishing and maintaining a latent infection, presenting approximately 3-log less reactivation-competent viruses than the WT virus. While D2-infected mice still displayed a minor latent virus load, with the latent viruses just above the limit of detection, the D2L92A latent viruses were below the limit of detection. During the long-term latency, the behavior of D2 and D2L92A viruses was the total opposite of the M2L92A virus, meaning there were no reactivation-competent viruses at day 50 pi. Since the D2L92A virus has a mutation in the H2-Kd-restricted T cell epitope, it would be expected to induce a long-term latent load with latent virus numbers similar to M2L92A ones at day 50 pi. In contrast, at day 50 pi, the latent viruses were below the limit of detection, with no apparent long-term latency.

To confirm that the results obtained for each virus reflected a truly latent infection, spleen samples were analyzed for presence of pre-formed infectious viruses. To do so, replicate samples were subjected to freeze-thawing, which disrupts the cells,

making impossible reactivation from latency but does not inactivate pre-formed viruses. Replicating viruses were then detected by incubation with permissive fibroblasts and examined for the presence of cpe. As can be seen in Figure 3, no pre-formed infectious viruses could be detected at any time point for any of the analyzed viruses.

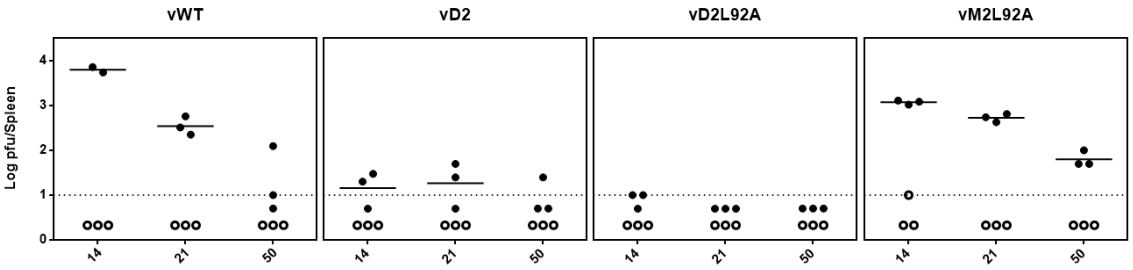

Figure 3. Recombinant MuHV-4 viruses display a strong latency deficit. Mice were infected intranasally with $1x10^4$ pfu of the indicated viruses. At 14, 21 and 50 days post-infection, spleens were removed and titrated for reactivation-competent virus by explant co-culture with BHK-21 cells. Each point represents the titer of an individual mouse. The horizontal bars show arithmetic means. The dashed horizontal line represents the limit of assay detection.

4.4 Mutation mLANA-D2 does not affect its nuclear localization

mLANA has a putative nuclear localization signal (NLS) in residues 21 to 37 (KRRCFNKPAAMPPKRRR) and, like kLANA, is a nuclear protein (Rodrigues *et al*., 2009, Ballestas *et al*., 1999, Habison *et al.*, 2012). It localizes primarily to the nucleus when transiently expressed in HEK 293T (Rodrigues et al., 2009). To assess if the introduced mutation affected the cellular localization of mLANA-D2, A20 cells were transiently transfected with either wtmLANA or mLANA-D2 encoding plasmids fused with GFP, and localization was assessed by immunofluorescence. Results showed that mLANA-D2, similarly to wtmLANA, localizes to the nucleus of transfected cells (Figure 4).

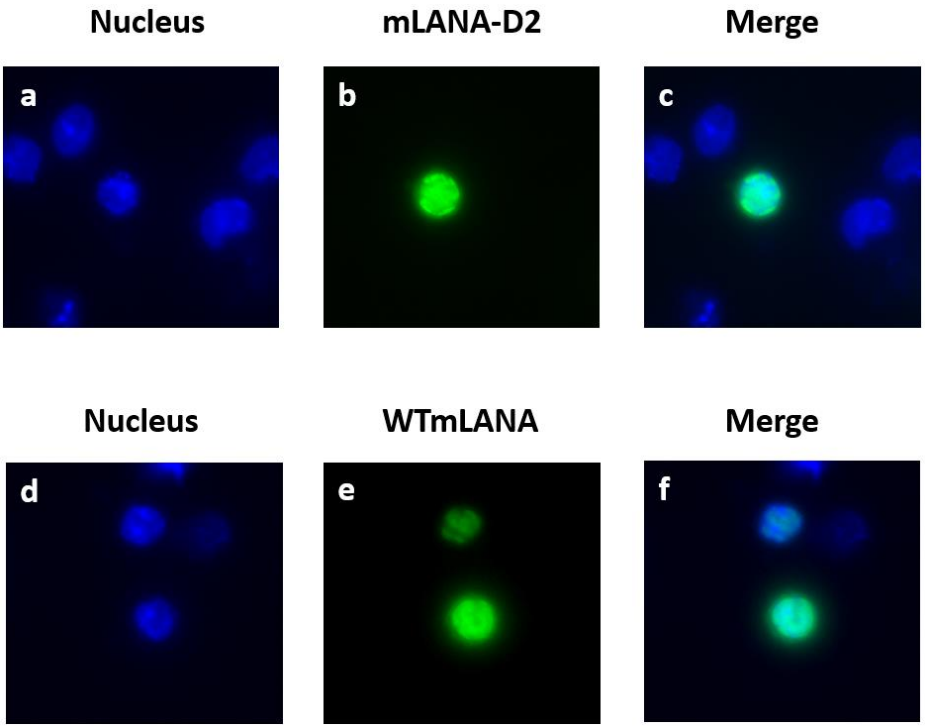

Figure 4. mLANA-D2 localises to the nucleus. A20 cells were transiently transfected with a plasmid encoding mLANA-D2-GFP (panels a,b and c) or wtmLANA-GFP (panels d,e and f). After 24 h, cells were incubated in fixative solution and permeabilised. Nuclear DNA was stained with DAPI solution (blue) to visualize the nucleus.

4.5 Expression of mLANA mutants

To further investigate if mLANA recombinant viruses were expressing the viral protein and that the phenotype exhibited in the infectious center assay was not due to a deficit in mLANA expression, BHK-21 cells were infected with WT virus and recombinant versions at a MOI of 2,5 pfu/cell and immunblotting was performed with the appropriate antibodies. Following 24 h of infection against mLANA and α-actin antibody, which serve as a loading control for cell infection, all four viruses expressed mLANA in infected fibroblasts (Figure 5). Both D2L92A and M2L92A viruses express mLANA equivalently, being D2 the virus less expressing the viral protein.

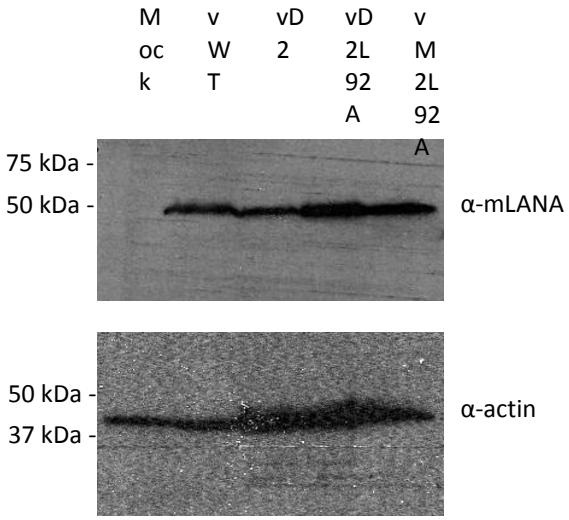

Figure 5. Expression of mLANA mutants is equivalent. BHK-21 cells were infected with WT MuHV-4 and recombinant versions at a MOI of 2.5 pfu/cell. After 24 h, of infection, mLANA and α-actin levels were analyzed by Western blotting using the indicated antibodies (first and second panels). Molecular weight (in kDa) is indicated on the left.

5. DISCUSSION

During this project, two recombinant viruses – vD2 and vD2L92A, were generated in order to evaluate the oncogenic role of MYC during gammaherpesvirus infection. They were cloned as bacterial artificial chromosomes (BACs) since this technique allows the maintenance of viral genomes by a BAC in *E.coli* and mutagenesis of the virus genome using the bacterial recombination machinery (Adler *et al*., 2003). The integrity of mLANA mutations was assessed by sequencing and the stability of the viral genomes cloned as BAC was verified by restriction digestion and compared with MuHV-4 BAC. Afterwards, infectious viruses were reconstituted by transfecting the BAC-DNA into BHK-21 cells and were subjected to sub-cultivation throught Cre-recombinase-expression fibroblasts to remove the BAC vector sequences, since the presence of the BAC vector sequences can attenuate virus growth *in vitro* compared to the WT (Adler *et al*., 2001). In the MuHV-4 BAC, the BAC sequences are flanked by loxP sites and the expression of Cre recombinase from the cellular genome can efficiently excise the BAC sequences during growth.

During the generation and characterization of recombinant viruses, revertant viruses should have been generated too in order to ensure that the eventual phenotype alterations in vD2 and vD2L92A viruses were due to desired mutations and not due to mutations accidentally introduced elsewhere in the viral genome. The phenotype of such revertants should be restored to wild type status.

Both recombinant viruses display normal growth *in vitro*, having a replication kinetics equivalent to WT, indicating that introduced mutations did not affected lytic replication. The results are in agreement with former studies showing that mLANA is not essential for *in vitro* replication of MuHV-4 (Fowler *et al*., 2003; Moorman *et al*., 2003). Likewise, M2 was also demonstrated to be dispensable for lytic replication (Jacoby *et al*., 2002; Macrae *et al*., 2003; Simas *et al*., 2004).

Being mLANA a nuclear protein (Rodrigues *et al*., 2009), it has a putative nuclear localization signal in residues 21 to 37 and, although the mutation introduced in mLANA-D2 was not in the NLS, it could modify the structure of the protein and prevent its nuclear localization. The results showed that the introduced deletion did not affect the nuclear localization of mLANA-D2 as can be assessed by the immunofluorescence assay in transiently transfected A20 cells.

The experiment was engineered with mLANA-D2 encoding plasmids fused with GFP, which, nevertheless, provides information regarding the possible disruption of

mLANAD2 NLS during the generation of recombinant virus. However, it would be more interesting to assess the nuclear localization of vD2 in the infected splenocyte population, using an antibody against the viral protein, mLANA.

The recombinant viruses were able to express mLANA as it was verified by the immunoblotting in BHK-21 cells. There were some expression differences between the viruses but the overall result showed that the introduced mutations did not affected the *in vitro* protein expression and that the phenotype of the infectious center assay was not due to the viral protein not being expressed. For the next step it would also be interesting to investigate the expression of mutated mLANA in the infected splenocyte population as well, in order to see the expression of the viral protein in a more physiological context. This last step would ensure the integrity of the recombinant viruses.

Since both mLANA and M2 proteins are especially required for latent infection during MuHV-4 pathogenesis, the recombinant viruses were analyzed *in vivo*. In the infection center assay, vD2 and vD2L92A display a major deficit in splenic latency, incapable of establishing and maintaining a latent infection. As complement to the *ex vivo* assay, spleen samples were analyzed for the presence of pre-formed viruses. As can be seen in Figure 3, pre-formed infectious virus, as measured by the parallel titration of equivalent frozen-thawed samples, was below the limit of detection, indicating that the splenic infection was solely latent. This phenotype could be the result of several factors.

Being mLANA essential for episome maintenance and persistence, the D2 mutation may have created a not functional latency protein, making it loss the episomal maintenance capacity.

Moreover, since both mLANA and kLANA share an extensive similarity and homology in the C-terminal region (Correia *et al*., 2013) it is possible that the N-terminal region of mLANA has a histone binding property similar to kLANA (Barbera *et al*., 2006). The D2 mutation could have resulted in recombinant viruses' inaptitude to attach to host chromosomes, impairing the ability to maintain latency during infection. The ability of mLANA mutants to interact with H2A histone was tested by immunoprecipitation experiment, a method used to study protein-protein interactions (data not shown). The result was inconclusive. Further immunoprecipitation conditions must be developed in order to test interaction of such proteins.

It is known that MYC is essential for the initiation and maintenance of GC (Calado *et al*., 2012; Dominguez-Sola *et al*., 2012) and that MYC deregulation can lead to opposites effects, such as tumorigenesis events which contribute to a variety of human cancers and anti-tumorigenic responses, such as apoptosis (Larsson & Henriksson, 2010). Since D2 mutation increases E3 ubiquitin-ligase activity of mLANA which results in stabilization of MYC, its overexpression could have led to a mutation in the germinal center or to a response as apoptosis, resulting in a deficit of splenic latency.

As a complement to the *ex-vivo* assay, limiting dilution analysis of virus-genome-positive cells in total splenocytes combined with real-time PCR should have been determinated, since one can argue that plaque assay was not sensitive enough to detect infectious recombinant MuHV-4 viruses or that the *ex-vivo* assay alone does not permit to deduce if the deficit is in latency or in reactivation from latency.

The assay consists of single cell suspensions obtained from pools of three spleens/virus which then are serially diluted 2-fold and 12 replicates of each cell dilutions are lysed overnight. The next day, samples are analyzed by real-time PCR with primer-probe sets specific for a viral protein. For each cell dilution, the number of negative PCR reactions, corresponding to a failure to obtain an amplification curve during the PCR cycles is determined. The frequency of virus DNA^+ cells is then calculated according to the single-hit Poison Model by maximum likelihood estimation. This model assumes that one limiting cell of one cell subset is necessary and sufficient for generating a positive response.

Since both vD2 and vD2L92A did not induced lymphoproliferation with further potentiating the genesis of tumor development, and could not establish a latent infection, a new approach must be developed to reveal molecular pathways critical for virus-induced lymphoproliferation. However it would also be interesting to identify mLANA and histones binding motifs, to shed some light in the deficit of latency.

6. CONCLUSION

This project allowed the exclusion of two recombinant viruses, vD2 and vD2L92A, as a way to study the oncogenic role of MYC modulation during gammaherpesvirus infection. Mutation at mLANA N-terminal region and mutation in a H2-k^d-restricted T cell epitope display a major deficit in splenic latency, incapable of establishing and maintaining a latent infection. Hence, vD2 and vD2L92A are not useful in future experiments and other approaches must be developed. Recombinant viruses capable of sustaining a latent infection must be engineer. Moreover, experiments to explore regions within the N-terminus of mLANA that interact with host histones must be exploited. Such results could help to understand the molecular pathways of Kaposi's sarcoma-associated herpesvirus latency.

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